Adult non-biting midges: possible windborne carriers of *Vibrio cholerae* non-O1 non-O139

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Summary

***Vibrio cholerae* is a waterborne bacterium native to the aquatic environment. There are over 200 known serogroups yet only two cause cholera pandemics in humans. Direct contact of human sewage with drinking water, sea-born currents and marine transportation, represent modes of dissemination of the bacteria and thus the disease. The simultaneous cholera outbreaks that occur sometimes in distant localities within continental landmasses are puzzling. Here we present evidence that flying, non-biting midges (Diptera; Chironomidae), collected in the air, carry viable non-O1 non-O139 serogroups of *V. cholerae*. The association of *V. cholerae* with chironomid egg masses, which serve as a *V. cholerae* reservoir, was further confirmed. In simulated field experiments, we recorded the transfer of environmental *V. cholerae* by adult midges from the aquatic environment into bacteria-free water-pools. In laboratory experiments, flying adult midges that emerged from *V. cholerae* (O1 or O139) contaminated water transferred the green fluorescent protein (GFP)-tagged pathogenic bacteria from one laboratory flasks to another. Our findings show that aerial transfer by flying chironomids may play a role in the dissemination of *V. cholerae* in nature.**

Introduction

The aquatic bacterium, *Vibrio cholerae,* comprises over 200 characterized serogroups, two of which (O1 and O139) are associated with cholera epidemics and pandemics (Shimada *et al.*, 1994; Kaper *et al.*, 1995; Colwell, 1996; Sack *et al.*, 2004). Other serogroups are associated with local outbreaks of cholera-like disease (Sharma *et al.*, 1998; Dalsgaard *et al.*, 2001). There is concern that new pathogenic, pandemic-causing strains may emerge from the environment (Faruque *et al.*, 2001, 2003; Sack *et al.*, 2004), as was the case of *V. cholerae* O139 (Faruque *et al.*, 2003). *Vibrio cholerae* survival in the environment and means of distribution of environmental and pathogenic strains are under study in several laboratories (Louis *et al.*, 2003; Sack *et al.*, 2003). Colwell and coworkers (see Huq *et al.*, 1983; Colwell, 1996) confirmed the hypothesis that the reservoir of *V. cholerae* is in the environment. They emphasized the importance of zooplankton components in the estuarine and other freshwater ecosystem, with special reference to copepods. Recently it was suggested that chironomid egg masses (Diptera; Chironomidae) serve as one of the natural reservoirs of *V. cholerae* (Broza and Halpern, 2001; Halpern *et al.*, 2004).

Chironomids, the non-biting midges (Diptera; Chironomidae), are the most abundant macro-invertebrate group in number of species and individuals encountered in freshwater aquatic habitats (Armitage *et al.*, 1995), and especially in estuaries (Benke, 1998), in which new cholera outbreaks first appeared (Colwell, 1996). Chironomids are closely related to mosquitoes (Culicidae), but female chironomids do not bite or feed. They undergo complete metamorphosis of four life stages: eggs, larvae, pupae (all of which are aquatic) and adults that emerge into the air. Females of *Chironomus* sp. lay egg masses at the water's edge. Each egg mass contains 400–2000 eggs, embedded in a thick, gelatinous matrix (Nolte, 1993.). The presence of several thousand egg masses at one site is not unusual and in extreme cases it may form layers of several centimetres thick along the water's edge (Nolte, 1993; Broza *et al.*, 2000, 2003).

Adult midges emerge from the pupal stage at the surface of aquatic localities and create swarms near or above water bodies to mate. The swarms may be driven by winds and are carried away from the original water body from which chironomid adults emerged. Some winged chironomids are highly mobile and are among the most common representatives of insect orders trapped at significant heights above land and far out at sea. For example see the reports on chironomids caught over the Mexican Gulf

and the Indian and Pacific Oceans (Glick, 1939; Bowden and Johnson, 1976; Sparks *et al.*, 1986).

Broza and Halpern (2001) have noted a complete destruction of an assemblage of egg masses immediately after removal from a natural habitat. The cause of egg mass degradation, that was followed by the failure of the eggs to hatch, was identified as *Vibrio cholerae* serogroup O9. An extracellular factor responsible for the degradation of chironomid egg masses was purified from *V. cholerae* O9 and O139 and was identified as a haemagglutinin/protease (HA/P) (Halpern *et al.*, 2003). In a further study, *V. cholerae* non-O1 and non-O139 were isolated from chironomid egg masses throughout the main annual season and from different freshwater bodies in Israel (Halpern *et al.*, 2004), indicating that the association between chironomids and cholera bacteria found earlier was a common coincidence.

Here, we report the isolation of environmental serogroups of *V. cholerae* from flying adults. In simulated field experiments and in laboratory experiments we demonstrated the potential of those insects to carry and transfer both pathogenic and non-pathogenic serogroups of this bacterium between water bodies. The potential of adult midges to disseminate the cholera bacteria is discussed.

Results

Field survey: adult midges as carriers of environmental Vibrio cholerae

Non-biting midge adults were caught regularly during March–October 2001, in two sites neighbouring waste stabilization ponds (WSP) in Israel, using light traps. Collection of egg masses from the water in nearby ponds was done simultaneously, using Styrofoam boards. Environ-mental (non-O1/non-O139) *V. cholerae* were readily isolated and identified from both adults and egg masses (Table 1). Somatic antigen serogrouping identification (Shimada *et al.*, 1994) was employed on ~50% of the *V. cholerae* isolates by E. Arakawa at the National Institute of Infectious Diseases, Tokyo, Japan (Table 1). Other sites were sampled occasionally between June 2000 and June 2004. Egg masses were collected from the water of 10 additional sites in Israel, India and Africa (Table 2). Altogether, 12 different serogroups of *V. cholerae* were identified from bacteria isolated from adults, of which seven were isolated also from egg masses collected at the edge of nearby water (Table 2). Most of the serogroups isolated from adults were re-isolated and identified more than once, indicating that their occurrence was not accidental.

Vibrio cholerae was isolated from both male and female adult chironomids (Tables 1 and 3). Adults that were caught on 24 July, 2001, were sorted into males, pre-oviposited females, and post-oviposited females (Table 3). The isolation of *V. cholerae* from those three groups demonstrates that the contamination is prior to oviposition and not dependent on sex (Tables 1 and 3). From the assemblage that was caught on the same night and same trap, two serogroups (O8 and O10) were isolated from both males and females. This implies that the whole assemblage of adults, participating in the mating swarms, has the potential to serve as an aerial vector of the bacterium.

The procedure of *V. cholerae* isolation was obtained with 10 adults incubated in 5 ml of alkaline peptone water (APW). This procedure enables the assessment of adult contamination. For example, 17 out of 21 attempts to isolate *V. cholerae* from a group of adults were successful (Table 3). Assuming that at least one adult was contami-

Table 1. *Vibrio cholerae* serogroups isolated from chironomid adults and egg masses collected in waste stabilization ponds in Israel during May and August, 2001.

Region	Date		Adults	Egg masses
Tivon, north Israel	May 14	♀♀ + ♂♂	O79	
	May 23	♀♀ + ♂♂	O10	
	May 29	–	–	O23, O184, O179
	June 6	♀♀ + ♂♂	O128	
	June 11	–	–	O2, O12, O27, O39
	June 15	1 male; emerged in lab	O41	
	June 25	♀♀ + ♂♂	O23, O79,	O10, O27, O70
	June 27	♀♀ + ♂♂	O83	
	July 10	♂♂	O79	O8, O34, O34, O109
		♀♀	O23, O79	
	July 17	♀♀	O23, O122	
	July 24	♂♂	O8, O10, O10	O23, O23
		♀♀ pre-oviposition	O8, O8	
		♀♀ post-oviposition	O10, O10	
Shafdan region, central Israel	June 13	♀♀ + ♂♂	O62	O124
	July 2	♀♀ + ♂♂*	O2	
	August 12	♀♀	O23	

*Viable *V. cholerae* were isolated from dead adults after 14 days at room temperature.

Table 2. List of all *V. cholerae* serogroups isolated and identified from chironomid egg masses and adults in all sites studied by us (data overlap some published data from Halpern and colleagues, 2004).

	Egg masses		Adults	
Country	Serogroups	Total no.	Serogroups	Total no.
Israel	O2, O3, O8, O9, O10, O12, O23, O27, O34, O39, O51, O70, O79, O94, O99, O109, O120, O122, O124, O140 O179, O184	22	O2, O8, O9, O10, O23, O41, O43, O62, O79, O83, O122, O128	12
Kenya	–[a]		O2[b]	1
Malawi	O128	1	–[a]	
Zanzibar	O10, O37, O49, O140, O201	5	–[a]	
India	O3, O13, O39, O78, O166	5	–[a]	

a. No research was performed.
b. Adults isolated from a swarm of a mixed non-biting midge population of the families Chaoboridae and Chironomidae.

Table 3. Isolation of *V. cholerae* from males, pre-oviposited females and post-oviposited females adults.

	No. captured per sampling night	Repeats of *V. cholerae* isolation[a]	Success in isolation of non-O1	No. samples that were serogrouped	Identification of serogroups
Males	140	10	8/10	3/8	O8, O10, O10
Females; pre-oviposition	208	5	5/5	2/5	O8, O8
Females; post-oviposition	322	6	4/6	2/4	O10, O10

a. 10 adults per 5 ml APW.

nated in each group where the bacteria were successfully isolated from, there is a rate of no less than 8% infected adults in the whole adults population. Preliminary results suggest that on adults caught from the environment, the *V. cholerae* infection load is 92.5 ± 97.4 cfu per adult.

Additional observations on the capacity of adults to transfer Vibrio cholerae

In August 2001, adult chironomids were captured with light traps 3 km away from a water source (Dan Region Waste Plant, south of Tel Aviv). *Vibrio cholerae* serogroup O23 was isolated from one group of 20 adult chironomids, representing the isolation of *V. cholerae* from airborne adult midges trapped at a distance from their aquatic habitat. This is complementary to the data presented so far (Table 1) that is based on trapping the adults on the water edge.

An independent and nationwide mosquito monitoring system has been in practice for the last couple of years, performed by the Central Laboratory, Ministry of Health, Israel. In the course of this programme, during the second half of 2003 and 2004, chironomid adults were sorted out from light traps and tested by the Central Laboratory for *Vibrio*, Ministry of Health (L. Lerner, per. comm.). *Vibrio cholerae* non-O1/non-O139 were isolated from chironomid adults, in 10 out of the 15 localities tested, those sites represent various geographical regions and types of water bodies (see Table 4). In six localities the traps were positioned without direct connection to water bodies (date plantation, etc.), demonstrating the potential for chironomid adults to carry environmental *V. cholerae* outside the aquatic environment (Table 4).

Table 4. Additional sampling sites of chironomids, from which *V. cholerae* were isolated (performed by L. Lerner, The Ministry of Health, Israel).

Region (Israel)	Neighbourhood	Site of trap	Sampling date (2003)
Desert oasis	Village, Nat. park and public centre	Palm plantation	July 1
		Palm plantation	July 7
		Palm plantation	October 2
	Village	Irrigated area	November 6
Semi-arid	Village	Sewage pond	July 15
Mediterranean zone	Village	Springs and stream	August 24
	Natural habitat	Stream	August 24
	Natural habitat	Stream	August 29
	City	Pond (rehabilitated water)	September 24
	Tel Aviv	Old farm	October 20
	Rural area	Artificial fish ponds	12 April–1 June, 2004

To date, more than 300 isolates of *V. cholerae* belonging to 35 different serogroups of non-O1/non-O139 *V. cholerae* have been isolated from chironomid adults and egg masses collected in Israel, India and Africa (Table 2).

Demonstration of aerial transfer in field experiments

Three control 180 cm diameter pools covered with both mosquito net and bird net (3/8 inch knotless) and three exposed (experimental) pools covered with bird net only, were deployed 50 m from a WSP in northern Israel. The double netting bordered an upper and lower boundary to the diameter of the aerial vector of *V. cholerae.* The pools were filled with clean drinking water and were monitored for the presence of chironomids and *V. cholerae*. We found that chironomid females attended the uncovered pools and laid their egg masses on their perimeters. In the first monitoring (day 2) 3–8 chironomid egg masses per pool were attached to the pool perimeter and several (5–10) adult carcasses were floating on the water. The appearance of chironomids in the uncovered pools was accompanied by the isolation of *V. cholerae* from the laid egg masses as well as from the water itself.

The presence of *V. cholerae,* in the net-covered and exposed pools, was monitored in day 0, 1, 2, 3, 7 and 9 according to the World Health Organization protocol for *V. cholerae* isolation. *Vibrio cholerae* presence in the water was recorded in the exposed pools after 2 days but was not detected at all in the net-covered pools – demonstrating that *V. cholerae* was transmitted by an organism larger than the mosquito net holes. The test was set first in July 2002 and repeated three times during the summer of 2003 and 2004. In each and every experiment conducted during 2002–04, *V. cholerae* presence in the experimental pools was detected in a 250 ml water sample taken between 48 h and 72 h after the initiation of the experiment. In parallel, the control, mesquite net-covered pools were clean of *V. cholerae* presence.

The minimal water volume that was needed for the detection of *V. cholerae,* by the procedure used here, was decreasing with time (Fig. 1), indicating that *V. cholerae* was transferred into the clean water and its numbers increased. This increase was most likely due to both bacterial multiplication and additional transfer of new bacteria by the constant flow of adult chironomids from the air. Detection of *V. cholerae* in the experimental pools was significantly different from the control pools in day 2, 3, 7 and 9 when 250 ml of water were sampled (One sample *t*-test, $P < 0.05$).

Low numbers of few other species of flying insects were counted in the uncovered pools upon the first pupation of the chironomid larvae, 10 days after the initiation of the experiment and 7 days after the successful isolation of
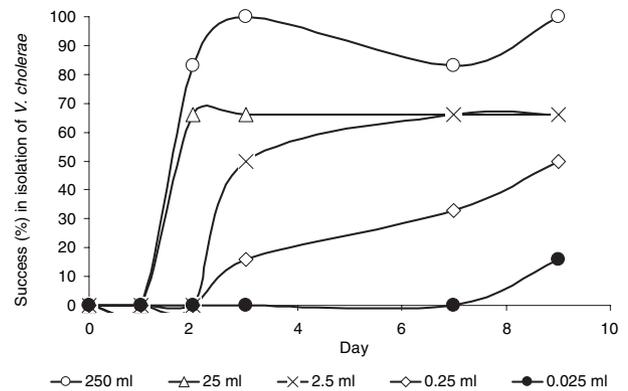
Fig. 1. Success of *V. cholerae* isolation from drinking water in rigid wall pools by date and volume of sample. During the late summer of 2004, six rigid wall pools were deployed and filled with fresh drinking water (0.5 m^3 each). Three control pools were covered with both mosquito net and bird net and three exposed (experimental) pools were covered with bird net only. All the pools were proximity to a WSP in northern Israel. In day 0, 1, 2, 3, 7 and 9 water samples of different volume (250 ml, 25 ml, 2.5 ml, 0.25 ml and 0.025 ml) were taken from each pool. *Vibrio cholerae* isolation procedure was carried out on each sample. *Vibrio cholerae* was undetectable in the control pools through the whole course of the experiment. Each line represents the success (%) of isolation of *V. cholerae* from a specific volume of water during the experimental period. Sampling 250 ml in day 2, 3, 7 and 9 was significantly different from the control (one sample *t*-test, $P < 0.05$).

V. cholerae from the pool's water). In one repeat, adults of the following two species were counted in the uncovered pools: the aquatic bug, *Anisops sardea* (Notonectidae) and the aquatic beetle *Laccobius* sp. (Hydrophilidae). They were checked for attached *V. cholerae* with negative results.

A laboratory simulation: adults as carriers of Vibrio cholerae

To further demonstrate the vector potential of adult chironomids, a laboratory qualitative simulation was conducted in which chironomid transfer *V. cholerae* isolates, both environmental as well as pathogenic O1 and O139 isolates, was performed. The bacteria were tagged with a green fluorescent protein (GFP)-encoding plasmid. The GFP-tagged bacteria were introduced (10^6 cfu ml^{-1}) into a flask containing midge larvae in the forth instar that were expected to pupate in 18–72 h. Adults subsequently emerged from the pupae and were captured from the air. *Vibrio cholerae* were readily detected on those adults. Vigorous agitation of the captured adults in APW and enumeration of total GFP-tagged *V. cholerae* found on those adults gave an average cfu of 8.9 ± 4.6. This low number is probably due to the fact that one cfu represents more than one bacterium attached to the insect. Checking the fluorescent bacteria on the adults revealed that most of them remained attached to the midge chitin and include

an assemblage of few bacteria each. Some of the adult females laid egg masses in the water of an external vessel and GFP-tagged *V. cholerae* was isolated from that water indicating transport by the contaminated chironomid adults. *Vibrio cholerae* O1 and O139 were indistinguishable in these experiments from *V. cholerae* O9 with respect to the ability to be carried by the chironomid adults and the location of the tagged bacteria on the adults.

Pockets of GFP-tagged *V. cholerae* were identified by fluorescent microscopy on the outer surface of adult midges, most frequently on intersegmental membranes (Fig. 2), a surface where one segmental membrane overlaps another and provides a microbial attachment site that is relatively protected from the environment. Other parts of the adult were occasionally contaminated with the fluorescent bacteria (Fig. 3), including part of the male external genitalia that grasp the female during copulation (Fig. 3, bottom), and the leg pulvilum (Fig. 3, top).

Discussion

In this study the vector potential of adult chironomids was demonstrated. In the field, *V. cholerae* isolates belonging to different environmental serogroups were identified on male and female chironomid adults that were caught in the air. Simultaneously, the presence of the cholera bacteria was observed on egg masses in the water (Tables 1–3). Few adults that carried *V. cholerae* were caught in a

distance from water body. Chironomid invasion from the air into bodies of water was observed using artificial pools. *Vibrio cholerae* appearance and presence was established soon thereafter. In the laboratory, it was shown that the pathogenic, clinical serogroups (O1/O139) were as readily transferred by the adult chironomids as the environmental isolate (O9).

Environmental Vibrio cholerae *and cholera epidemics*

As cholera has not occurred in Israel since the large pandemic of 1970, it is no surprise that *V. cholerae* isolated in our field study were of the non-O1/O139 serogroups. Nevertheless, both trips to India (February 2001) and Zanzibar Island (February 2002) lasted 2 weeks only and were carried out in distinct inter-epidemic period, timing in which the O1/O139 serogroup were supposed to be very rare. The distribution of *V. cholerae* has been associated with sea currents, limnological events, and water macrophyte and non-insect invertebrates (Lipp *et al.*, 2002). Our findings show an additional link between *V. cholerae* and insects, in particular the chironomids.

The different serogroups listed in Table 1 reveal high diversity within a population of a bacterium at a site. Its ecological meaning is yet unravelled. The relevance of research on non-O1 serogroups in the current and future epidemiology of cholera is related to the assumption that some of the environmental serogroups 'could be a proto-
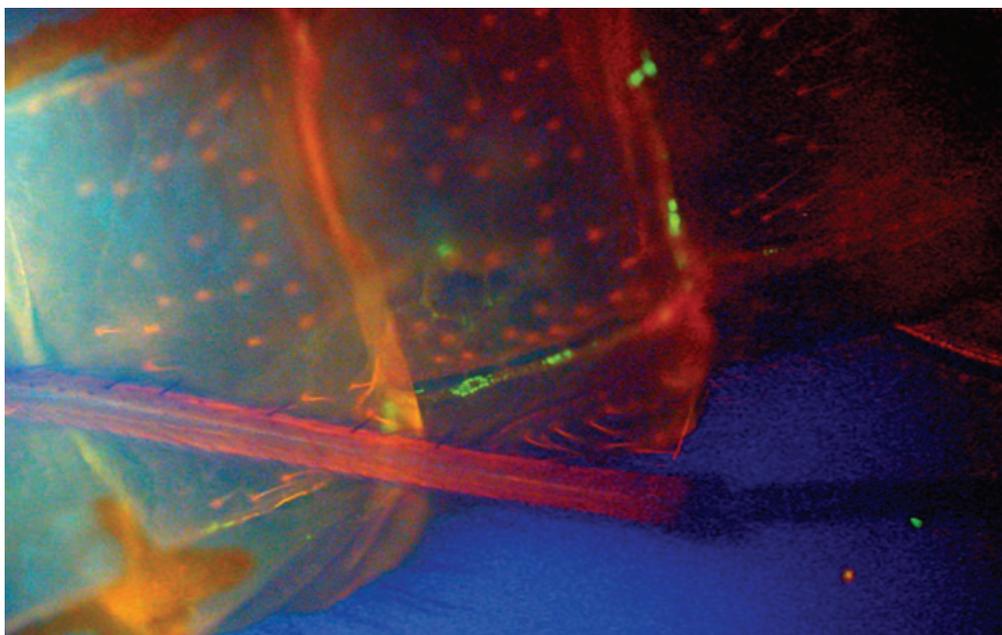
Fig. 2. *Vibrio cholerae* O9 tagged with GFP, located on the intersegmental membrane of an adult chironomid exoskeleton. The adult chironomid emerged from a vessel in which chironomid larvae were incubated with *V. cholerae* serogroups O9, O1 or O139 tagged with GFP, for ~24 h. The chironomid was examined under an epifluorescent microscope. A dorsal view of three abdominal segments; the one in the middle partially hides a distinct chain of fluorescent bacteria on the lateral intersegmental membrane. Two shiny clusters are seen on the intersegmental membrane of its posterior end (×100).
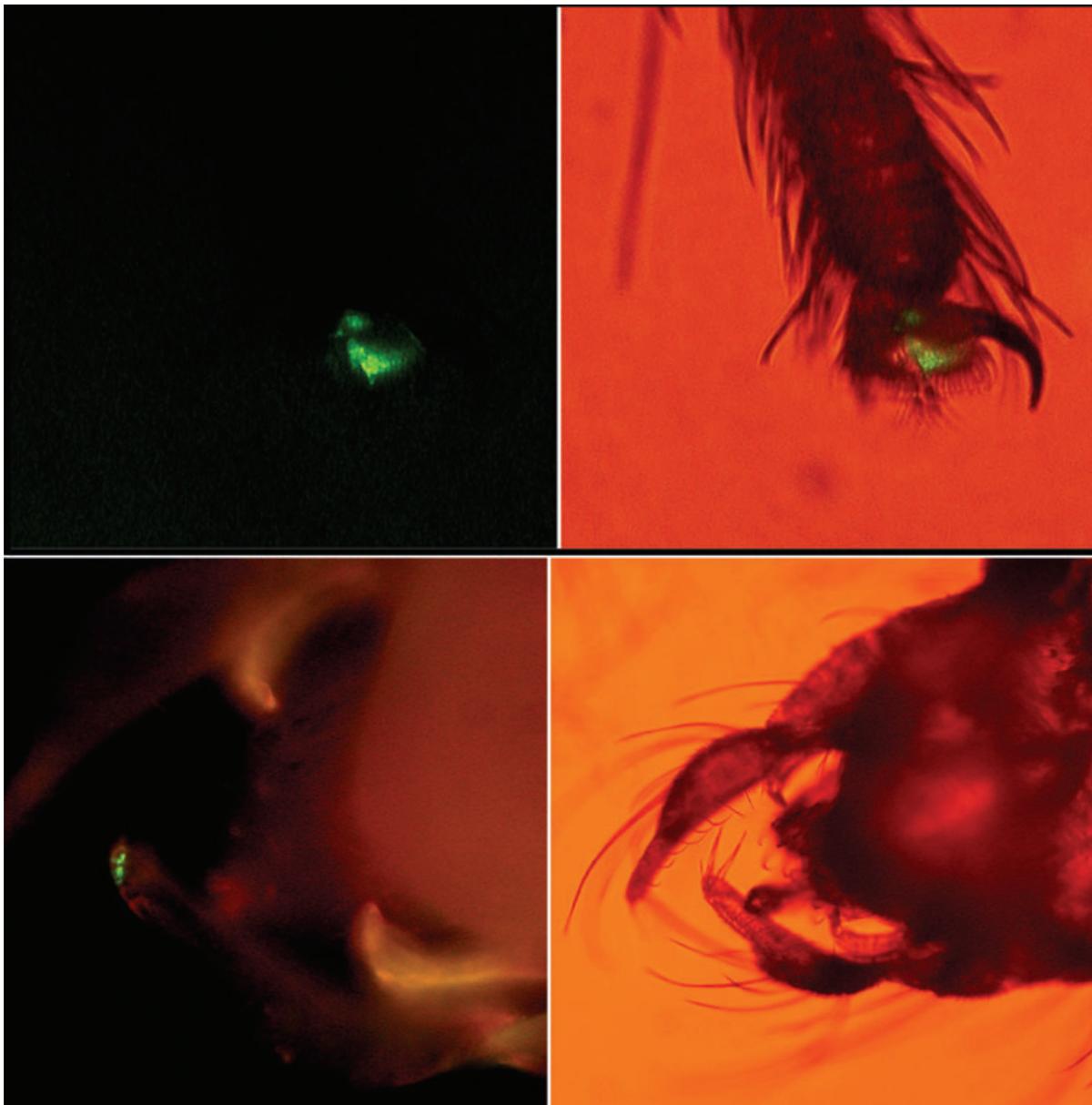
Fig. 3. *Vibrio cholerae* tagged with GFP on adult chironomids. Adult chironomids emerged from a vessel in which chironomid larvae were incubated with *V. cholerae* tagged with GFP for ~24 h. The chironomid was examined under a microscope with fluorescent light (left panel) and visible light (right panel).
Top: *V. cholerae* O1 tagged with GFP on the tarsal pulvillum of an adult chironomid leg (×400).
Bottom: *V. cholerae* O1 tagged with GFP on male external genitalia (×200).

cholera agent' (Sharma *et al.*, 1998). Apparently this was shown during the new outbreak of O139 in Daka, Bangladesh 2002 (Faruque *et al.*, 2003). It is claimed that serogroup O139 is a consequence of a horizontal transfer of genes from the environmental serogroup O22 into *V. cholerae* O1 El-Tor. In order to allow such a horizontal transfer to exist both serogroups must share the same habitat, the same physical pool which is also the same gene pool (Faruque *et al.*, 2004; Sack *et al.*, 2004). Therefore, our observations on chironomid and environmental

V. cholerae connection are highly relevant to the cholera outbreaks.

Chironomid adults as carriers of Vibrio cholerae

The sites on adult's body where the GFP-tagged bacteria were seen most frequently are the intersegmental membranes, which are relatively protected from erosion (Butt and Goettel, 2000). The adherence of *V. cholerae* to chitin and the role of surface proteins in the process have been

demonstrated (Tarsi and Pruzzo, 1999) and may explain the mechanism by which these bacteria are transported by adult non-biting midges. Not less frequent is the attachment of bacteria to the special pads of Diptera legs, the pulvilum (Fig. 3), known by its sticky fatty secretion. *Vibrio cholerae* was isolated even from the pulvilum of non-aquatic Diptera such as the housefly *Musca domestica* (Fotedar, 2001). Although adults of aquatic bugs (Notonectidae) and beetles (Hydrophilidae) colonized the pools in our experimental simulation, we did not detect *V. cholerae* on them. We assume that this is not always the case. We may expect that other flying insects that spend part of their time on water surface may also carry these bacteria. Shukla and colleagues (1995) isolated both O1 and non-O1 *V. cholerae* from water striders (bugs) of the genus *Gerris* caught on the Ganga River near Varanasi. We may assume that those water striders may sometimes deliver *V. cholerae* from one source of water to adjacent one, by active flight. Even so, with regard to fast and massive transmission of infectious disease as cholera, their potential seems limited. Water striders are large insects that occur in low numbers compared to chironomids. They mate on water surface without creating swarms and are rare in the aero-plankton (see for example Glick, 1939). In contrast, chironomids are ideal candidates for *V. cholerae* dissemination. The linkage of both *V. cholerae* and chironomids, in space and time is very clear; both are very common in euthrophic water bodies including man-made polluted water. In many areas of endemic infection, cholera epidemics occur seasonally (Pascual *et al.*, 2000; Colwell and Huq, 2001), in the same pattern as chironomids do (Vandebund and Groenendijk, 1994; Halpern *et al.*, 2004). The swarming behaviour of the chironomid adults gives another dimension for their vector potential. In Israel, during the 1998 chironomid seasonal peak, it was estimated that 2.5×10^9 adults emerged each night per square-kilometre from Dan Region Waste Stabilization ponds (Broza *et al.*, 2000). Even if only 8% of those adults (Table 3) have *V. choleare* attached to their body, still 2×10^8 adult per square-kilometre will be carrying the bacterium. They create huge swarms for mating, and during the mating or soon thereafter many of those adults were seen driven by winds off the ponds carrying environmental *V. cholerae* with them.

Aerial transport of Vibrio cholerae*: how far?*

The observation reported here suggests that adult chironomids are carriers of environmental *V. cholerae* strains that attach to their body. Those adults are shown to transfer *V. cholerae* between close water bodies. How far, and whether this route is a major route of cholera dissemination, are questions for future research. In order to explore such hypothesis other tools and different scale and experiments should be carried out. Yet, the following facts support this path of research: winged dipteran insects, including chironomids, are highly mobile and are among the most common representatives of insect orders trapped on high elevation and far out at sea (Armitage *et al.*, 1995). They have been found in significant number (Reynolds *et al.*, 1999) at 150 m above ground in West Bengal, India, the heart of the endemic site of cholera. We isolated viable *V. cholerae* from dead adults even 14 days post emergence (note in Table 1) and we assume that they remain viable even during long distance transfer.

The next step in this research should include a study that would be carried out during an active cholera epidemic. The presence of pathogenic isolates (O1/O139) of *V. cholerae* on chironomids in an endemic area can lead to an immediate improvement of monitoring of new outbreaks and prediction of its patterns of distribution.

Experimental procedures

Chironomid taxonomy

The population of *Chironomus* inhabited in the two waste plants mentioned in the current research, includes sympatric population of three species, partially not overlapping along the temporal scale. The main species was identified by J. Martin (CSIRO, Australia 2004) as *Chironomus transavalensis*, an East African species. The second species is *Chironomus* sp. and the third belongs to a third Chironomini species. In another water habitat, during June 2004, *V. cholerae* was isolated from *Chironomus plumosus* egg masses (M. Broza and N. Meltser, unpubl.)

Chironomid collection

Samples of chironomid adults and egg masses were collected regularly from the area of the city of Tivon, northern Israel, and sporadically in various other sites as noted. Flying insects were caught by light traps (J.W. Hock Company, Gainesville, FL 32604, USA) and transferred to a sterile vessel. Chironomid adults were manually identified and separated. Chironomid egg masses were collected from styrofoam plates (0.2 m × 0.2 m or 0.25 m × 0.25 m) deployed in the monitoring ponds 18 h before the sampling.

Isolation of presumptive Vibrio cholerae *from chironomids*

Samples of chironomid adults and egg masses were incubated in APW for 6 h (viable *V. cholerae* can be isolated from adult chironomids up to 2 weeks after capture). A 10 µl loopful of broth was streaked on thiosulfate citrate bile sucrose agar (TCBS agar, Becton-Dickenson, Franklin Lakes, NJ, USA). The plates were incubated overnight at 37°C. Suspected yellow, flat, 1–3 mm diameter colonies were picked from each sample and streaked on Luria–Bertani (LB) agar containing 1% NaCl and incubated overnight in 37°C. Samples were transferred to Carry–Blair agar stabs before transport of samples for serogrouping.

Adult's infection load

Ten adults were incubated in 1 ml APW (four replicates) for 20 min at 37°C, after which samples of 100 μl were spread onto TCBS agar. Suspected yellow colonies were counted and picked from each plate. The identity of *V. cholerae* was confirmed as detailed below. Note that the results did not include the bacteria that remained adhered to the adult's body.

Confirmation of presumptive Vibrio cholerae *isolates*

Presumptive isolates of *V. cholerae* were confirmed by biochemical tests and serogrouping or by polymerase chain reaction (PCR). Suspected colonies picked from LB agar were characterized with API-20E kits (bioMérieux, France). *Vibrio cholerae* strains were subjected to serogroup analysis, using O serogroup-specific antisera (by E. Arakawa, Department of Bacteriology, National Institute of Infectious Diseases, Tokyo, Japan). Biochemical identification was confirmed by PCR using specific primers for *V. cholerae* targeted to the 16S–23S rRNA intergenic spacer region (Chun *et al.*, 1999) or targeted to the outer membrane protein gene OmpW (Nandi *et al.*, 2000). Polymerase chain reaction products were subjected to electrophoresis using 1.5% (w/v) agarose gel to resolve the amplified products, which were visualized under UV light after ethidium bromide staining.

Strains and plasmids

pSMC2 in *Escherichia coli* DH5alpha was a gift from Dr Kolter (Watnick *et al.*, 1999). pVSP61TIR in *E. coil* JM109 was a gift from Dr Lindow (Miller *et al.*, 2000). *Vibrio cholerae* strains: *V. cholerae* of serogroups O1 Inaba and O1 Ogawa obtained from L. Lerner (Government Central Laboratories, Jerusalem, Israel). *Vibrio cholerae* O139 obtained from T. Ramarmurthy (National Institute of Cholera and Enteric Diseases, Calcutta, India).

Bacterial growth and plasmid purification

All strains were grown on LB medium at 37°C. Where appropriate, the culture medium was supplemented with antibiotics at the following concentrations: ampicillin 80 μg ml⁻¹, kanamaicin 30 μg ml⁻¹. *E. coli* harbouring the relevant plasmid was grown overnight in LB containing the appropriate antibiotic. Plasmids were purified using a plasmid purification kit (QIAprep miniprep kit, QIAGEN, Hilden, Germany) and kept at (−20°C) until use.

Electroporation

Electroporations were performed as described by Hamashima and colleagues (1995). After electroporation, cells were grown for 1 h, with agitation, at 37°C and streaked on plates emended with antibiotic. Green, fluorescent colonies were isolated.

Laboratory transfer experiment

An experimental apparatus was assembled from three glass instruments: a 100 ml Erlenmeyer flask in a 1 l beaker were inserted into a 5 l tall beaker (glassware from Schott Duran, Mainz, Germany). The apparatus was covered with a net and autoclaved before use. The insect larvae (in 50 ml of water) were transferred to the Erlenmeyer and, soon after, the bacteria suspended in the same water were introduced into the Erlenmeyer. A total of 200 ml of water was poured on the perimeter of the 5 l beaker. Erlenmeyers without bacteria or without larvae were used as controls. The adults after emerging were drawn to the light at the lid of the beaker in a way that their return to the water was virtually impossible. Adult flying chironomids were removed from the outside beaker after 5 min incubation in −20°C. Isolation of presumptive *V. cholerae* from the outside water and from the adult chironomids was done as above.

Microscopy

Epifluorescence microscopy was done with a Zeiss Axioscop II equipped with a UV Lamp (Nikon). Digital pictures were taken with a Nikon Coolpix 995 digital camera.

Field transfer experiment

Artificial, rigid-walled, pools (SA-7730, Aqua-Leisure Industries, Avon, MA 02322 USA) were deployed on a concrete platform 50 m from the monitoring pond. A fibreglass net (18 × 14 mesh, wire thickness 0.011 inch, Cat. no. 71125, New York Wire, Mt. Wolf, PA, USA) in two overlapping layers was used to cover the control pools, while the other pools (experiment pools) was partially (≈10%) covered with the same net. The pools were filled with fresh drinking water. A total of 200 g of sea sand was autoclaved and added to each pool. The water and insects in the pools were monitored with sterile instruments. Samples of water (250 ml, 25 ml, 2.5 ml, 0.25 ml and 0.025 ml), and chironomids, when detected (as soon as 24 h after the pools were deployed and throughout the entire monitoring period), were collected and filtered by using 0.45 μm pore-size membrane filters (HAWG047S1, Millipore Watertown, MA 01730, USA). This was followed by incubation of the membranes in an enrichment medium consisting of 5 ml of APW (1% peptone, 1% NaCl; pH 8.0) overnight at 37°C. Two loopfuls of the culture broth, taken from the top layer of the APW, were streaked onto TCBS agar and incubated overnight at 37°C. Yellow, flat, 1–3 mm diameter colonies were picked from each sample and streaked on LB agar containing 1% NaCl. In the two last repeats of these experiments all the pools were covered with a bird net (3/8 inch knotless) to serve as an upper limit for the dimensions of the aerial vector.

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